EXHIBIT 2

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ORIGINAL REPORT

Detecting BRCA2 Protein Truncation in Tissue Biopsies to Identify Breast Cancers That Arise in *BRCA2* Gene Mutation Carriers

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The Acknowledgment and Appendix are included in the full-text version of this article; they are available online at www.jco.org. They are not included in the PDF version (via Adobe® Reader®).

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ABSTRACT

Purpose
Mutations in the *BRCA2* gene are dominantly inherited but cause cancers when the wild-type allele has loss of heterozygosity (LOH) within the cancer. Because most disease-associated *BRCA2* mutations are protein-truncating mutations, a test for truncated BRCA2 proteins should identify most *BRCA2* hereditary cancers.

Methods
We have developed a tissue truncation test to identify truncated BRCA2 proteins in breast cancer tissue biopsies in vivo that does not use amplification or genetic manipulations. N-terminal and C-terminal antibodies are used to visualize protein truncation by demonstrating that the beginning of the protein is present but the end (ie, terminus) is absent.

Results
A quantitative C-terminal immunostaining score or a C-terminal to N-terminal truncation ratio correctly classified 20 of 21 breast cancers arising in *BRCA2* mutation carriers and 57 of 58 cancers arising outside the context of a multiple-case breast cancer family. This represents a sensitivity of 95% and a specificity of 98%. Because of the presence of C-terminal BRCA2 protein and atypical clinical features of the misclassified cancer in a *BRCA2* mutation carrier, we performed polymerase chain reaction and sequence analyses on this cancer. The results showed continued presence of the *BRCA2* wild-type allele in the cancer, which indicated that intact BRCA2 protein was present in this cancer.

Conclusion
This immunohistochemistry-based test (which takes only 4 hours) appears to identify *BRCA2* hereditary cancer with high accuracy. The test also appears to diagnose the biochemical loss of BRCA2 protein in cancers (ie, *BRCA2*-mutant genotype), which will usually but not always agree with the presence of a germline *BRCA2* mutation found by susceptibility testing by DNA sequencing of blood samples.

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INTRODUCTION

Germline mutations in the *BRCA2* gene are dominantly inherited and are generally thought to cause cancer after somatic loss of the wild-type allele within the cancer.[1-5] Because most disease-associated *BRCA2* mutations are protein-truncating mutations,[6-7] a test for truncated BRCA2 proteins should identify most *BRCA2* hereditary cancers. It is important to know which breast cancer patients have cancers arising in *BRCA2* mutation carriers, because they have a much greater risk of subsequent breast cancer recurrence or development of ovarian cancer than patients with sporadic cancer.[8-9] Identification of these gene mutation carriers also is important because family members who inherited mutations have a lifetime breast cancer risk of 35% to 80%,[10] and *BRCA2* breast cancers respond differently to specific treatments.[11-12] Present strategies for finding *BRCA2* mutation carriers are cumbersome and are only applicable in high-risk families in whom less than half the mutant cancers are found.[13]

We have developed an antibody-based method to identify truncated BRCA2 proteins in breast cancer specimens. This method successfully classified 20 of 21 breast cancers from patients in whom the cancers were arising in *BRCA2* mutation carriers; this method may represent a useful new screening method to identify these patients. Comparison of N-terminal and C-terminal BRCA2 immunostaining correctly

classified 57 of 58 cancers arising outside the context of a multiple-case breast cancer family.

METHODS

Generation of C-Terminal Monoclonal Antibody

We generated a monoclonal BRCA2 antibody by using the C-terminal peptide 3,284 to 3,294 of sequence TFVSPAAKAGG. This peptide was conjugated to keyhole limpet hemocyanin and was used to immunize mice. As an initial test of the immune response, we screened immunized mice by enzyme-linked immunosorbent assay (ELISA) and then tested the highest-titer mouse sera by performing immunohistochemistry (IHC) on MCF7 cell pellets and cancer samples; we also used the antisera for Western blots. Two mice were chosen for splenectomy and cell fusion on the basis of the specificity for C-terminal BRCA2 protein by using the immune response initial testing strategy. Clonal supernatants were screened by Western blotting of cell samples and by IHC on a patient with breast cancer arising in a *BRCA2* mutation carrier (ie, in a separate patient not included in the 21 patients with breast cancer for the study) and on another breast cancer from a patient with no family history of cancer. The best clones were selected by choosing those that were high titer by ELISA, that detected a single 220-kDa band on Western blot, and that differentiated the breast cancer arising in a *BRCA2* mutation carrier from the other breast cancer sample cleanly. On the basis of this strategy, clone 575A15 was selected and cloned, and the supernatant from the cell line was affinity purified.

Western Blotting of Proteins

MCF7 cells were lysed in radioimmunoprecipitation assay buffer and were standardized for equal protein, and then samples were separated by

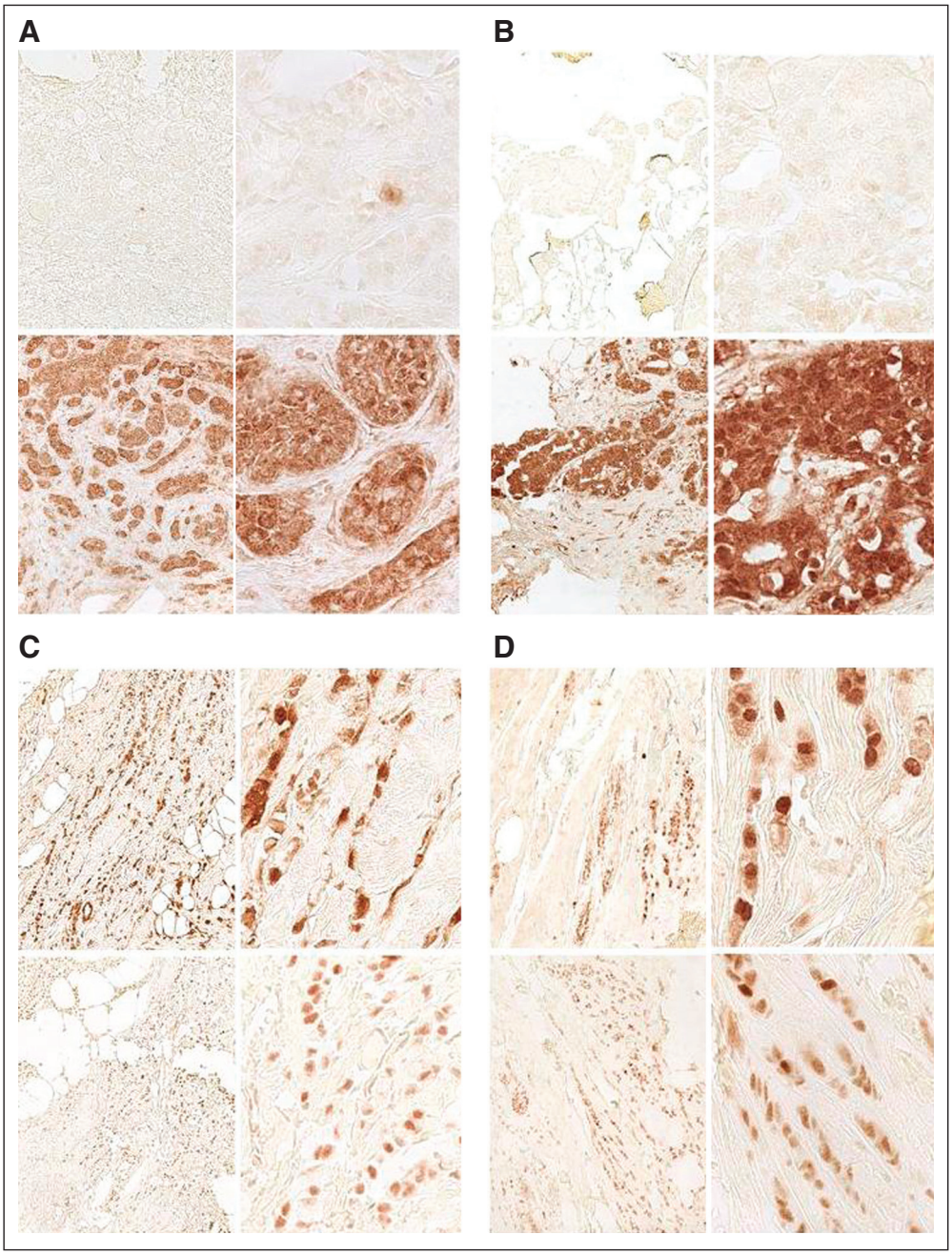

Fig 1. Dual C-terminal and N-terminal BRCA2 immunohistochemistry (IHC) of hereditary breast cancers (A and B, respectively) and of sporadic breast cancers (C and D, respectively). Magnification is (left) ×20 and (right) ×100 for all panels. The upper panel for each pair is C-terminal IHC, and the lower panel is N-terminal IHC. (A) Samples are from a *BRCA2*-mutant cancer with 7231del5. Top panel stained with C-terminal BRCA2 antibody, and second panel stained with N-terminal BRCA2 antibody. Only a lymphocyte in the top panel stains. (B) Samples are from a patient with a 9654delTT *BRCA2* mutation at similar magnifications and similar staining. (C and D) The paired panels are from adjacent sections of the same sporadic breast cancer sample. Note the similar nuclear staining with both antibodies for the sporadic cancer samples.

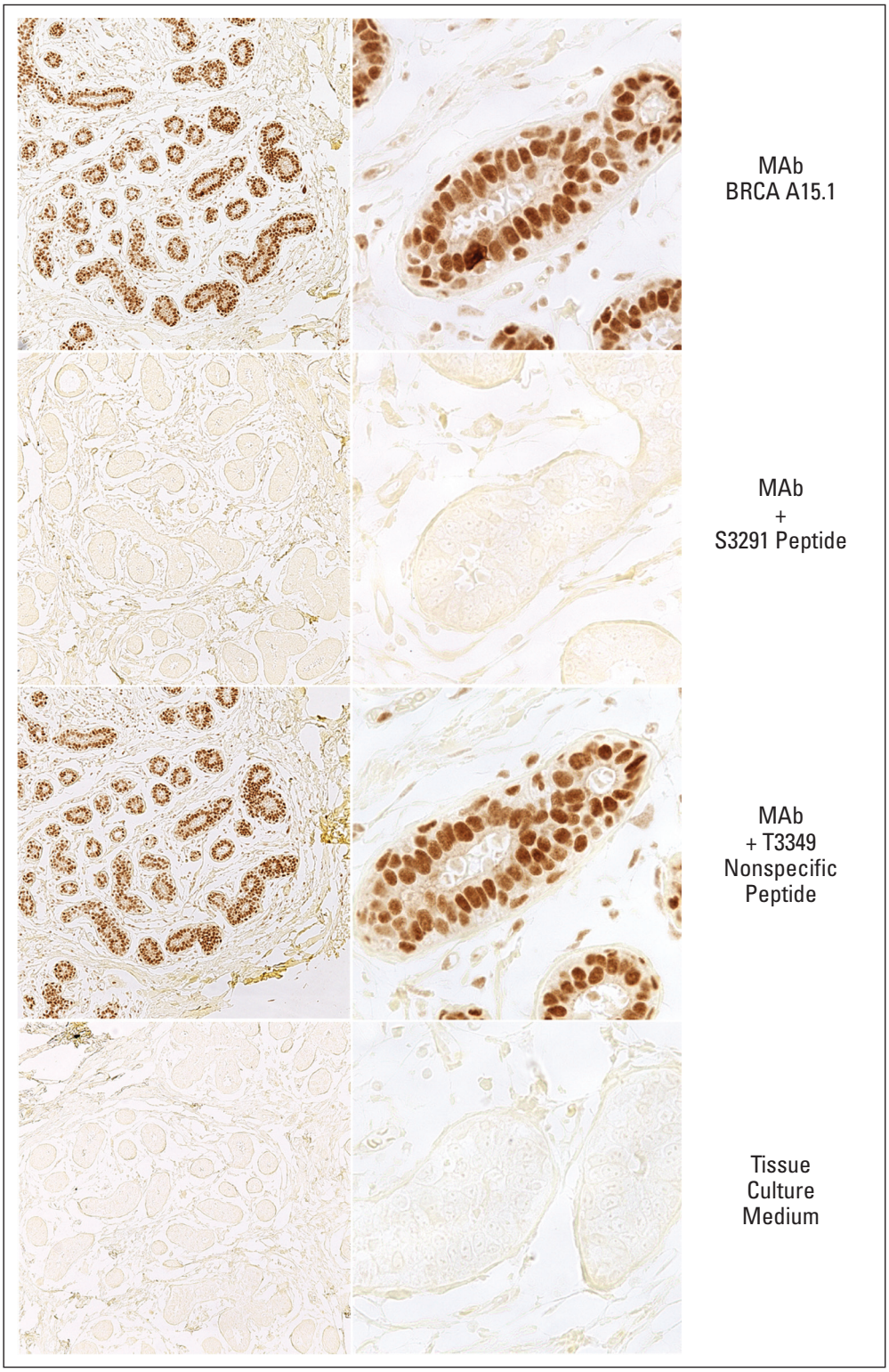

Fig 2. Immunohistochemistry with 575A15 C-terminal monoclonal antibody on normal breast epithelial lobules. Upper panels: untreated antibody. Middle panels: antibody mixed with excess of immunizing peptide (BRCA2 amino acids 3284 to 3294: TFVSPAAKAGG). Lower panels: antibody mixed with excess of control BRCA2 peptide (BRCA2 amino acids between 3300 and 3400, obtained from Abcam (Cambridge MA). Magnification is (left) ×20 and (right) ×100.

Tris/acetate sodium dodecyl sulfate polyacrylamide gel electrophoresis on 3% to 8% Novex NuPage mini-gels (Invitrogen, Carlsbad, CA) for 1 hour and 160 volts. Proteins then were transferred to polyvinylidene difluoride membranes at 30 volts for 1 hour (Figs 1 and 2) in Tris/glycine transfer buffer that contained 8% methanol at 16°C. Membranes were blocked for 1 hour at room temperature in 5% dry, nonfat milk in phosphate-buffered saline (PBS). The primary 575A15 monoclonal antibody was incubated overnight at 4°C and was diluted 1:500 in 0.5% dry, nonfat milk/PBS-Tween 0.1%. After appropriate washes, antirabbit–horseradish peroxidase (GE Healthcare/Amersham Biosciences, Pittsburgh, PA) that was diluted

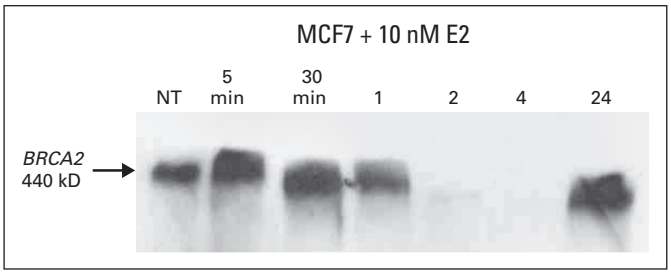

Fig 3. MCF7 cells were cultured for 48 hours in 10% charcoal-stripped serum/phenol red–free DMEM and were treated with 10 nmol/L estrogen for 5 and 30 minutes and for 1, 2, 4, and 24 hours. Samples were blotted with the 575A15 BRCA2 C-terminal monoclonal antibody. The 440 kDa band is the only band on the blot.

1:5,000 in 0.5% dry nonfat milk/PBS-Tween 0.1% was added and was incubated for 1 hour at room temperature. Detection was performed with ECL Plus reagents (GE Healthcare/Amersham Biosciences).

IHC

IHC analysis was performed on 4-micron sections of formalin-fixed, paraffin-embedded tissue. After deparaffinization and rehydration, heat-induced antigen retrieval was achieved in a 20 mmol/L citrate buffer at pH 6.0 for 10 minutes in a decloaking chamber (Biocare Medical, Concord, CA), and this was followed by a 25-minute, room temperature cooling period. Endogenous peroxidase was suppressed by incubating sections for 10 minutes in 3% hydrogen peroxide/PBS. Sections then were treated with a blocking solution (DAKO serum-free protein block; DAKO, Carpenteria, CA) for 1 hour. The N-terminal antibody used was from R&D systems MAB2476 (a commercially available mouse monoclonal antibody directed against an *E coli*–derived recombinant human BRCA2 protein that spans amino acids 1-200), which was used at 3 μg/mL. The C-terminal antibody was the newly generated mouse monoclonal 575A15 that is described in Methods and that was used at 4 μg/mL. Sections were incubated with the primary antibodies in a humidified chamber overnight at 4°C, were washed in buffer, and were treated with a horseradish peroxidase–labeled polymer detection system (DAKO Envision Plus, Dual Link) for 30 minutes at room temperature. Peptide block studies were performed by preincubating the C-terminal monoclonal antibody with a 50-fold excess of immunizing peptide antigen or a control peptide and staining as indicated. The chromagen used was DAKO DAB Plus (brown) for both the C-terminal and N-terminal antibodies. The cytoplasmic yellow counterstain was Metanil Yellow (ScyTek Laboratories, Logan, UT). The IHC results were read with the pathologist blinded to the carrier status of the cancer patients.

PCR Amplification of Tissue Samples and Genotyping by Restriction Digestion or DNA Sequencing

Paraffin sections on single slides were microdissected by our published method,[14] were purified by xylene and ethanol extraction, and underwent proteinase K treatment and purification on a Qiagen column (Qiagen, Valencia, CA). PCR primers 5641F: 5′ ATGAAGATATTTGCGTTGAGGA and 6224R: 5′ CACTTGTCTTGCGTTTGTAATG were used to amplify DNA from tissue samples of normal breast, and normal and cancer samples from patients 13 and 21 were microdissected to obtain relative pure-normal versus cancer-cell populations. Amplified DNA then was purified on a Qiagen column and was restricted with the enzyme Hin4I, and samples were analyzed on a 2% agarose gel. PCR-amplified DNA also was used as a template for DNA sequencing with the nested primers 5753F: 5′ TGCATTTAGGATAGCCAGTG and 6000R: 5′ GAATGTCAGCAAAAACCTTAT.

RESULTS

Testing for protein-truncating mutations in patient DNA is a frequently used approach for breast cancer gene mutation screening. This is most commonly done by amplifying patient DNA with PCR and then assaying for truncation by in vitro translation—a test called the Protein Truncation Test.[15] The goal in this research was to develop a method for identifying truncated proteins without DNA amplification or genetic manipulations of the tissue. Because there is a polymorphic DNA sequence variant found in 1% of the population in the C-terminus of BRCA2 that is not associated with breast cancer,[16] we selected an epitope (amino acids 3284-3294) for immunization that would not identify that polymorphic truncated protein. Because of this design, all truncated BRCA2 proteins identified in breast cancers that used this antibody should be disease associated. To visualize protein truncation and to control for protein degradation or improper fixation, we employed an N-terminal antibody as a control to verify that full-length BRCA2 protein was present but truncated in breast cancers arising in *BRCA2* mutation.

We selected the highest-titer monoclonal antibody that showed specificity for C-terminal BRCA2 protein in ELISA, Western blots, and IHC. Figure 3 shows that the 575A15 monoclonal antibody detects a single 440-kDa protein by Western blotting, which shows the expected biphasic expression pattern after estrogen treatment of MCF7 cells. To test the specificity of the monoclonal antibody for IHC, we performed peptide blocks with both the immunizing peptide and a control BRCA2 peptide. The results show complete inhibition of nuclear IHC staining by the immunizing peptide but no effect of the control peptide (Fig 2), which indicated that the monoclonal antibody is highly specific for BRCA2 protein even in tissue sections.

To determine the sensitivity and specificity of this tissue truncation test for identifying *BRCA2* hereditary cancers, we tested breast cancer samples from 21 different patients with *BRCA2*-truncating mutations and 58 sporadic breast cancers. The mutations for each patient are listed in Table 1. Figure 1A provides an example of such a tissue truncation test by demonstrating strong N-terminal BRCA2 protein in the lower panels but absent C-terminal staining in the upper panels within cancer cells. A lymphocyte within the tumor in Figure 1A provides strong nuclear staining, because the biallelic gene inactivation found in the cancer did not occur in nonmalignant cells such as inflammatory cells, which thus provides an internal control for the IHC process. Appendix Figure A1 (online only) also provides data to show that *BRCA2* positivity is absent in tumors from mutations carriers but is present in adjacent normal breast tissue, because the wild-type allele is present. Figures 1B to 1D show protein truncation within cancers arising in *BRCA2* mutation carriers but not within cancers arising outside the context of a multiple-case breast cancer family that show similar N-terminal and C-terminal immunostaining. By scoring the IHC as 0, 1+, 2+, or 3+ for both antibodies, we can determine N-terminal and C-terminal scores. Tables 1 and 2 show that this tissue truncation test can distinguish breast cancers arising in *BRCA2* mutation carriers from likely sporadic breast cancers. The sensitivity for these tests for identifying these cancers was 95%, and the specificity was 98%.

Because *BRCA2*-mutant cancer sample 13 had atypical clinical features (ie, estrogen-receptor negative) and expressed abundant C-terminal BRCA2 protein (Table 1), we hypothesized that this might represent an atypical cancer for a *BRCA2* mutation carrier, which lacks the expected biallelic gene inactivation. Because inherited cancers normally undergo a somatic loss of wild-type BRCA2 sequence within the cancer, we PCR amplified this region of

Table 1. *BRCA2* Mutations Identified in Patients With Breast Cancer

Patient	*BRCA2* Mutation*	C-Terminal IHC	N-Terminal IHC	Truncation Ratio
1	6503delTT	0 and 0	2+ and 2+	0
2	W2586X	0 and 0	3+ and 3+	0
3	W2586X	0 and 0	3+ and 3+	0
4	IVS7 + 2T>G	0 and 0	2+ and 2+	0
5	IVS7 + 2T>G	0 and 0	2+ and 2+	0
6	9654delTT	0 and 0	2+ and 3+	0
7	7231del5	0 and 0	2+ and 2+	0
8	7231del5	0 and 0	2+ and 3+	0
9	7231del5	0 and 0	3+ and 3+	0
10	7231del5	0 and 0	3+ and 3+	0
11	6503delTT	0 and 0	3+ and 3+	0
12	1983del5	0 and 0	2+ and 2+	0
13	S1882X	3+ and 3+	3+ and 3+	1
14	5946delCT	0 and 0	2+ and 2+	0
15	5849del4	0 and 0	2+ and 2+	0
16	983del4	0 and 0	2+ and 2+	0
17	983del4	0 and 0	3+ and 3+	0
18	4075delGT	0 and 0	2+ and 2+	0
19	4075delGT	0 and 0	2+ and 3+	0
20	3945delA	0 and 0	3+ and 3+	0
21	S1882X	0 and 0	2+ and 2+	0

Abbreviation: IHC, immunohistochemistry.
*Breast Cancer Information Core database designation, National Human Genome Research Institute.

BRCA2 and then digested the amplified DNA with the restriction enzyme Hin4I, which digests the wild-type sequence but not the mutant-truncating 5873C→A DNA sequence. This occurs because Hin4I has a recognition sequence of $5'_{8}$(N)G A Py $(N)_5$ (A/C/G) T C $(N)_{13}$-3′, so the truncating mutation destroys the final C in the recognition sequence. Appendix Figure A2 (online only) shows that Hin4I digests normal tissue and patient 13 with cancer to a similar extent. However, cancer patient 13 has a 5873C→A mutation; however, in this instance, the enzyme does not digest tumor DNA, because the wild-type sequence is not present.

DISCUSSION

We have developed an IHC-based tissue truncation method to visualize protein truncations in *BRCA2* hereditary breast cancer. This method was tested with both breast cancers arising in *BRCA2* mutation carriers and sporadic breast cancers, and the method had a sensitivity of 95% and specificity of 98%. This strategy provides a direct demonstration that a truncating mutation is present within a sample, unlike a mere loss of C-terminal or internal immunostaining that could result from decreased expression, promoter methylation, protein degradation, or other reasons. Results listed in Table 2 demonstrate the importance of comparing IHC by using both antibodies, because analysis of only a C-terminal antibody would likely misclassify three samples (ie, patients 32, 48, and 57), which would reduce the test specificity to 93%.

There are two clear caveats for identifying *BRCA2* mutations by searching for truncated proteins: first, this method cannot identify missense mutations or DNA sequence variants of unknown significance; second, truncated proteins must be relatively stable or the N-terminal IHC also would be negative. Because many *BRCA2* missense variants have been identified as neutral (ie, not disease associated) and others have been identified as unknown significance (and therefore not reliable enough for genetic counseling at this time), this method should be able to rapidly identify most, but probably not all, disease-associated *BRCA2* mutations.[6,7] As missense mutations are identified that clearly are disease-associated, strategies need be developed to screen for these occurrences; this is also true for protein-truncation testing on DNA samples. If, in future samples, some truncated proteins are unstable, as has been reported,[17] then the truncation test might be confusing, because both C-terminal and N-terminal IHC would be diminished or zero. A cellular process known as nonsense-mediated decay often produces instability of mRNAs that contain truncating (ie, nonsense) mutations that lead to protein truncation. The amount of mRNA instability often varies on the basis of the distance from the nonsense codon to the beginning of the exon,[18] and this effect has reportedly decreased BRCA2 mRNA levels from 1.5-fold to four-fold.[19] However, this finding differs from studies that have analyzed protein levels in transfected cells[20] and that did not show marked decreases in protein levels in cells that expressed truncated BRCA2 proteins. We also did not observe decreased N-terminal IHC staining of BRCA2 proteins in the majority of patients, as listed in Table 2, although three patients with likely sporadic cancers did show 1+ staining with both N-terminal and C-terminal antibodies. Decreased total BRCA2 protein levels can be identified if *BRCA2* hereditary cancers are identified as a result of regulatory mutations or large deletions. A summary of the strengths and weaknesses of this IHC strategy is listed in Table 3.

Because of the simplicity of this IHC test, it may be completed within 4 hours of receipt of tissue blocks and also may be used on small amounts of archival material. Because this method identifies the molecular lesion responsible for *BRCA2* hereditary cancer (the loss of

Table 2. Immunostaining Scores for Sporadic Breast Cancer Samples

Patient	C-Terminal	N-Terminal	Patient	C-Terminal	N-Terminal
1	3 and 3	2 and 2	30	2 and 2	2 and 2
2	3 and 2	3 and 2	31	2 and 3	2 and 2
3	2 and 3	2 and 2	32	1 and 1	1 and 1
4	3 and 2	3 and 2	33	2 and 2	2 and 2
5	3 and 3	3 and 2	34	2 and 3	2 and 3
6	3 and 3	3 and 2	35	3 and 3	3 and 3
7	2 and 3	2 and 2	36	3 and 3	2 and 2
8	3 and 2	3 and 2	37	3 and 3	2 and 2
9	3 and 3	3 and 2	38	3 and 3	2 and 3
10	3 and 3	2 and 3	39	3 and 2	2 and 2
11	3 and 3	2 and 2	40	3 and 3	3 and 3
12	3 and 3	3 and 2	41	2 and 2	2 and 2
13	3 and 3	2 and 3	42	3 and 3	3 and 3
14	2 and 3	2 and 2	43	2 and 2	2 and 3
15	3 and 3	3 and 2	44	2 and 2	2 and 2
16	3 and 2	2 and 2	45	2 and 3	3 and 2
17	3 and 3	3 and 2	46	2 and 2	2 and 2
18	2 and 3	3 and 2	47	2 and 2	1 and 2
19	3 and 3	2 and 2	48	1 and 1	1 and 1
20	2 and 3	3 and 3	49	2 and 2	2 and 2
21	2 and 3	2 and 2	50	3 and 2	2 and 2
22	3 and 3	2 and 2	51	2 and 2	2 and 2
23	3 and 3	2 and 3	52	3 and 3	2 and 3
24	3 and 2	2 and 2	53	3 and 2	2 and 2
25	2 and 3	2 and 2	54	2 and 3	2 and 2
26	3 and 3	3 and 3	55	2 and 2	2 and 2
27	2 and 2	2 and 2	56	2 and 2	2 and 2
28	3 and 3	3 and 3	57	1 and 1	1 and 1
29	2 and 2	2 and 2	58	3 and 3	2 and 2

full-length functional BRCA2 protein), it is fundamentally a functional test that distinguishes cancers that lack wild-type BRCA2 protein from cancers that express wild-type BRCA2 protein. For this reason, these IHC results occasionally may differ from DNA sequence results if a sporadic-type cancer (without biallelic *BRCA2* inactivation) occurs in a patient with a germline mutation. Because breast cancer is a common disease, it probably is not surprising to find some breast cancers in *BRCA2* mutation carriers that appear to result from more sporadic breast cancer mechanisms rather than from the expected biallelic gene inactivation thought responsible for *BRCA2* hereditary cancer. The data in Appendix Figure A2 suggest that cancer in patient 13 is such an occurrence. Because loss of heterozygosity in this region of chromosome 13 occurs in approximately 20% to 30% of sporadic breast cancers without germline mutations of *BRCA2*, this type of analysis is only informative in instances in which the wild-type allele is lost. The truncation testing strategy in this study clearly misclassified this patient for the purpose of identifying mutation carriers and family screening (hence, our 95% sensitivity, because this patient was misclassified). However, it may have provided the correct result for selecting targeted therapies aimed at *BRCA2*-defective cancers. Additional work is necessary to determine how frequently these types of patients are identified and whether the germline mutation or the loss of intact BRCA2 protein is a better predictor of therapeutic response.

Table 3. Strengths and Weaknesses of Immunohistochemistry Truncation Test

Strengths	Weaknesses
Rapid, 4-hour turnaround	Cannot identify missense mutations
Excellent sensitivity and specificity	Nonsense-ediated decay could produce false negatives
More patients effectively screened	Does not identify the specific DNA mutation
Does not find missense variants	Possible false positives as a result of expression levels

An IHC test may be performed at most hospital laboratories and does not require complex or expensive instrumentation. Vaz et al[21] have reported a C-terminal IHC test to detect *BRCA1* mutations but have analyzed only a few patients and controls. IHC testing also may be performed on archived paraffin blocks that have been stored for some time. Several of our tested paraffin blocks were from patient samples that were more than 20 years old and still gave reliable data. One advantage of a successful IHC approach is that this could be done irrespective of family history, which is not a good indicator of carrier status, particularly in young women. The ability to perform a truncation test for hereditary cancer at time of biopsy and resection could be useful to identify appropriate therapies for these patients. *BRCA2*-mutant cancers reportedly have been more sensitive to ionizing radiation,[22] poly (ADP-ribose) polymerase inhibitors,[11-12] and cisplatin,[23] so more complete identification of hereditary breast cancers could facilitate the appropriate targeted therapy for these patients. Although this truncation test cannot be used directly to screen family members,

it does identify patients who likely have truncating mutations, so DNA sequencing of patients and subsequent screening of family members could be useful for cancer prevention.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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